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Reclassification of the *Candida haemulonii* Complex as *Candida haemulonii* (*C. haemulonii* Group I), *C. duobushaemulonii* sp. nov. (*C. haemulonii* Group II), and *C. haemulonii* var. *vulnera* var. nov.: Three Multiresistant Human Pathogenic Yeasts

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The *Candida haemulonii* species complex is currently known as *C. haemulonii* groups I and II. Here we describe *C. haemulonii* group II as a new species, *Candida duobushaemulonii* sp. nov., and *C. haemulonii* var. *vulnera* as new a variety of *C. haemulonii* group I using phenotypic and molecular methods. These taxa and other relatives of *C. haemulonii* (i.e., *Candida auris* and *Candida pseudohaemulonii*) cannot be differentiated by the commercial methods now used for yeast identification. Four isolates (*C. haemulonii* var. *vulnera*) differed from the other isolates of *C. haemulonii* in the sequence of the internal transcribed spacer (ITS) regions of the nuclear rRNA gene operon. The new species and the new variety have a multiresistant antifungal profile, which includes high MICs of amphotericin B (geometric mean MIC, 1.18 mg/liter for *C. haemulonii* var. *vulnera* and 2 mg/liter for *C. duobushaemulonii* sp. nov) and cross-resistance to azole compounds. Identification of these species should be based on molecular methods, such as sequence analysis of ITS regions and matrix-assisted laser desorption ionization–time of flight mass spectrometry.

Candida and *Aspergillus* species are the most common causes of invasive fungal infections in immunocompromised individuals, but besides these fungi, many other yeast species and filamentous fungi can be pathogenic in such individuals (7). The list of reported species that cause human infection is constantly growing, partly because of recent advances in molecular tools and diagnostics. Thus, new clinically relevant species such as *Candida metapsilosis*, *Candida orthopsilosis*, *Candida bracariensis*, and *Candida nivariensis*, have been described recently (1, 5, 39).

Candida haemulonii (van Uden and Kolipinsky) S. A. Meyer and D. Yarrow (41) (syn. *Torulopsis haemulonii*) is one of the rare yeast species that can be isolated from human clinical sources. The species originally described was from the gut of a blue-striped grunt fish (*Haemulon scirus*) in 1962 (40). The first isolation of this yeast from a human, i.e., from the blood of a patient with renal failure, was reported by Lavarde et al. (22). Since then, several cases of infections due to this yeast have been described in the literature, varying from superficial to deep infections. Cases of catheter-related fungemia (18), bloodstream infections (30, 34), and osteitis (6) and outbreaks in intensive care units (16) have been reported recently. The species has also been isolated from toenails of diabetic patients (13). Noteworthy is the susceptibility profile of this yeast, which shows high MICs of amphotericin B (AMB) and fluconazole (FLC) (ranges, 0.5 to 32 and 4 to >64 mg/liter, respectively), which can hinder the management of patients with deep infections caused by this yeast. This antifungal profile has often been associated with clinical failure (6, 16, 17, 30, 34).

The *C. haemulonii* species complex was further studied by Lehman et al. in 1993 (24). They studied 25 strains from different geographic origins and clinical sources and described two genetically distinct *C. haemulonii* groups, I and II. This classification was based on isozyme profiles, DNA reassociation experiments, and physiological characteristics.

In recent years, two species related to *C. haemulonii* have been described, namely, *Candida pseudohaemulonii* and *Candida auris*, which are phylogenetically closely related to *C. haemulonii* in the *Metschnikowiaceae* clade (20). In 2006, Sugita et al. described *C. pseudohaemulonii*, which was isolated from the blood of a Thai patient. This species is as resistant to AMB and azole agents as are the two genetic groups of *C. haemulonii* (38). The second related species, *C. auris*, was described in 2009 by Satoh et al. and isolated from the external ear canal of an inpatient in a Japanese hospital. Using sequence analysis of the D1/D2 domain of the 26S rRNA gene and the internal transcribed spacer (ITS) regions of the nuclear rRNA gene operon, it was found that the strain represents a new species with a close phylogenetic relationship to *C. haemulonii* (35). In 2009, 15 isolates of *C. auris* were recovered from the ear canals of patients suffering from chronic otitis media in South Korea. All of these isolates showed a reduced susceptibility to AMB and azole compounds (17). Therefore, it is important to identify these species correctly in order to provide optimal patient care.

Recently, *C. haemulonii* and closely related species have caused

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TABLE 1 Isolates and reference strains of *C. haemulonii*, *C. pseudohaemulonii*, and *C. auris* used in this study

Isolate	Species	Origin	Location
CNM-CL7256	*C. haemulonii* var. *vulnera*	Toenail	Alicante, Spain
CNM-CL7462	*C. haemulonii* var. *vulnera*	Patient (source unknown)	Spain
CNM-CL4642	*C. haemulonii*	Patient (source unknown)	Argentina
CNM-CL3458	*C. haemulonii*	Blood	Argentina
CNM-CL7829W	*C. duobushaemulonii*	Blood	Spain
CNM-CL7239^T	*C. haemulonii* var. *vulnera*	Skin wound	León, Spain
CNM-CL7829P	*C. duobushaemulonii*	Blood	Spain
CNM-CL6800	*C. haemulonii*	Skin wound	Elche, Spain
CNM-CL7793	*C. haemulonii*	Blood	Bilbao, Spain
CNM-CL4640	*C. haemulonii*	Patient (source unknown)	Argentina
CNM-CL7073	*C. haemulonii* var. *vulnera*	Skin wound	Alicante, Spain
CNM-CL4641	*C. haemulonii*	Patient (source unknown)	Argentina
CBS 7800	*C. duobushaemulonii*	Foot ulcer	Tennessee
CBS 7799	*C. duobushaemulonii*	Foot ulcer	Georgia
CBS 5149^T	*C.haemulonii*	Gut of *Haemulon scirus* (fish)	Florida
CBS 9754	*C. duobushaemulonii*	*Pyrrhocois apterus* (insect)	Ulm, Germany
CBS 7801	*C. haemulonii*	Toenail	Hawaii
CBS 5150	*C. haemulonii*	Seawater	Lisbon, Portugal
CBS 6590	*C. haemulonii*	Patient (source unknown)	France
CBS 5468	*C. haemulonii*	Seawater	Brazil
CBS 6915	*C. duobushaemulonii*	Unknown	Unknown
CBS 7798^T	*C. duobushaemulonii*	Foot ulcer	Alabama
CBS 7802	*C. haemulonii*	Foot ulcer	Rhode Island
CBS 6332	*C. haemulonii*	Blood	Kuwait
CBS 10973	*C. haemulonii*	Blood	Kuwait
CBS 10972	*C. haemulonii*	Blood	Kuwait
CBS 10971	*C. haemulonii*	Blood	Kuwait
CBS 10970	*C. haemulonii*	Blood	Kuwait
CBS 10969	*C. haemulonii*	Blood	Kuwait
CBS 10968	*C. haemulonii*	Blood	Kuwait
CBS 10004^T	*C. pseudohaemulonii*	Blood	Thailand
KCTC-17807	*C. pseudohaemulonii*	Blood	South Korea
KCTC-17808	*C. pseudohaemulonii*	Blood	South Korea
KCTC-17809	*C. auris*	Ear	South Korea
KCTC-17810	*C. auris*	Ear	South Korea

[a] A superscript T indicates the type strain.

outbreaks in South Korea and Kuwait (16, 17). The reasons for their emergence are not clear, but they may be related to selective pressure as a result of the commonly applied FLC or AMB therapy. *C. haemulonii* and *C. pseudohaemulonii* were isolated from patients with central venous catheter-related fungemia, whereas the *C. auris* strains were isolated from the ear canals of inpatients (28). Moreover, the first three cases of bloodstream infection due to *C. auris* have been described recently (23).

In the present study, the phylogenetic relationships among 30 isolates of the *C. haemulonii* complex from the collections of the Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre (CBS-KNAW), Utrecht, The Netherlands, and the Mycology Reference Laboratory of the National Center of Microbiology, CNM-ISCIII, Majadahonda, Madrid, Spain, were studied and compared with known *C. auris* and *C. pseudohaemulonii* isolates. Four strains of the latter species from South Korea were included. The profile of susceptibility of 30 isolates of the *C. haemulonii* complex to nine antifungal compounds was assessed to establish a correct antifungal susceptibility profile of these species, as well as their ability to produce biofilms. Identification of all species by amplified fragment length polymorphism (AFLP) analyses, ITS sequence data, and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is possible.

(This work was partially presented at the 22nd ECCMID 2012 in London, England.)

MATERIALS AND METHODS

***C. haemulonii* group I and group II isolates.** The 30 isolates of *C. haemulonii* used in this study are listed in Table 1. These include the type strain of *C. haemulonii* group I (CBS 5149^T) and two representative isolates of *C. haemulonii* group II (CBS 7798 and CBS 7799). Isolates from animal and environmental sources were included to make the sampling more robust. Four strains of the two closely related species *C. auris* and *C. pseudohaemulonii* (including type strain CBS 10004^T) were included for comparison.

DNA extraction. Genomic DNA was extracted from yeasts grown in glucose yeast peptone agar medium (GYPA) at 25°C. DNA extraction was performed by using the protocol described by Bolano et al., with slight modifications (2). After 2 days of incubation, a suspension of cells was added to 150 μl of sterile sand, 750 μl of lysis buffer, and 750 μl of phenol-chloroform (1:1, pH 8.0). This suspension was shaken at 2,500 rpm for 3 min, and the crude extract was centrifuged at 17,000 × *g* for 15 min at 4°C. The 700 μl of supernatant was transferred into a 1.5-ml Eppendorf tube. An equal volume of ice-cold 96% ethanol and 100 μl of 3

M ice-cold sodium acetate was added to the supernatant. The solution was mixed and stored for 30 to 60 min at −20°C. The DNA was pelleted at 17,000 × *g* for 10 min at 4°C. The supernatant was discarded, and the pellet was air dried. This pellet was resuspended in 100 μl of preheated Tris-EDTA buffer and incubated at 37°C and 65°C for at least 10 min.

Amplification and nucleotide sequence determination. The ITS and the D1/D2 regions of the ribosomal DNA were sequenced. The primer set V9-G/LR3-R was used to obtain the amplicon (11). For sequence analyses, primers ITS1 and ITS4 were used for the ITS region and NL1 (21) and LR3-R were used for the D1/D2 domain (Assembling the Fungal Tree of Life website, http://aftol.org/). The sequencing PCR was performed as follows. For each reaction mixture, 17.75 μl of MilliQ water, 0.75 μl of $MgCl_2$ (50 nM), 2.5 μl of PCR buffer 10x, 1.9 μl of a 1 mM deoxynucleoside triphosphate (dNTP) mixture, 0.5 μl of primer V9-F (10 pmol/μl), 0.5 μl of primer RLR3R-R (10 pmol/μl), 0.1 of *Taq* polymerase (5 U/μl), and 1 μl of DNA solution were used. The amplification reactions were done using a first cycle of denaturation for 5 min at 96°C, followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 52°C for 30 s, and elongation at 72°C for 1 min, with a final extension step of 5 min at 72°C.

The *RPB1* (which encodes the largest subunit of RNA polymerase II) and *RPB2* (which encodes the second largest subunit of RNA polymerase II) genes were analyzed as follows. For *RPB2*, the conditions and primers used for amplification and sequencing were described by Liu et al. (26). The amplicon was obtained with primers RBP2-5F (5′-GAYGAYMGWG ATCAYTTYGG-3′) and RPB2-7Cr (5′-CCCATRGCTTGYTTRCCCAT-3′). Because of the length of the amplicon (approximately 1 kb), the sequencing reaction was carried out with primers RPB2-5f and RPB2-7Cr and also with primers RPB26F (5′-TGGGGKWTGGTYTGYCCTGC-3′) and RPB26R (5′-GCAGGRACCAWMCCCCA-3′).

For the *RPB1* gene, primers RPB1af (5′-GARTGYCCDGGDCAYTTY GG-3′) and RPB1Cr (5′-CCNGCDATNTCRTTRTCCATRTA-3′) were used (Assembling the Fungal Tree of Life website, http://aftol.org/). A PCR mixture contained 17.75 μl of MilliQ water, 0.75 μl of $MgCl_2$ (50 nM), 2.5 μl of PCR buffer 10x, 1.9 μl of a 1 mM dNTP mixture, 0.5 μl of primer RPB1af (10 pmol/μl), 0.5 μl of primer RPB1Cr (10 pmol/μl), 0.1 μl of *Taq* polymerase (5 U/μl), and 1 μl of DNA solution. The following amplification conditions were used: a first cycle of denaturation for 5 min at 94°C, followed by 36 cycles of denaturation at 94°C for 50 s, annealing at 52°C for 60 s, and elongation at 72°C for 1 min, with a final extension step of 7 min at 72°C. For the sequencing reaction of the *RPB1* gene, the following volumes were used per reaction mixture: 4 μl of MilliQ water, 3 μl of dilution buffer, 0.5 μl of primer (10 pmol/μl), 1 μl of BigDye version 3.1, and 1 μl of diluted amplicon solution. The sequencing PCR conditions used were a first cycle of denaturation for 1 min at 95°C, followed by 30 cycles of denaturation at 95°C for 10 s, annealing at 50°C for 5 s, and elongation at 60°C for 4 min.

Sequencing products were purified with Sephadex (Amersham Pharmacia, The Netherlands). Both strands of purified gene fragments were sequenced at the Uppsala Genome Centre (Uppsala, Sweden). Sequences were assembled and edited with SeqMan II software (DNAStar Inc., Madison, WI) and aligned with MegAlign (DNAStar). The sequences were visually corrected. All phylogenetic analyses used maximum likelihood with 2,000 bootstrap simulations and were conducted with InfoQuest FP software, version 4.50 (Bio-Rad Laboratories, Madrid, Spain).

Identification by MALDI-TOF MS. For identification by MALDI-TOF MS, the full extraction method (ethanol-formic acid [FA]) of Marklein et al. was used, with slight modifications (27). Strains were subcultured on Sabouraud dextrose agar plates and incubated for ≤24 h at 30°C. An Eppendorf tube (1.5-ml volume) was filled with 0.3 ml of MilliQ water and 2 μl of yeast biomass (with a 1-μl sterile inoculation loop [Greiner Bio-One]). After vortexing, 0.9 ml of absolute ethanol was added and mixed vigorously for 1 min. Following centrifugation (17,000 × *g*, 2 min), the supernatant was discarded and the residual ethanol was removed by pipetting after a second centrifugation step, followed by air drying of the pellets at room temperature. The volume of 70% FA (Fluka, Zwijndrecht, The Netherlands) was then adjusted to the pellet size (usually 30 to 40 μl) and the material was first detached by pipetting and mixed by vortexing until the pellet was completely dissolved in FA. Then a volume of pure acetonitrile (ACN, Sigma-Aldrich, Zwijndrecht, The Netherlands) equal to the volume of FA was added, mixed by vortexing for 5 min, and then centrifuged (17,000 × *g*, 2 min). A 1-μl volume of the supernatant was pipetted onto a clean, polished steel target plate (Bruker Daltonik, Bremen, Germany). For each strain tested, two spots were prepared. Once the spotted material was air dried at room temperature, it was overlaid with 1 μl of HCCA matrix solution (α-cyano-4-hydroxycinnamic acid at 10 mg/ml; Bruker Daltonik, Bremen, Germany) dissolved in 50% (vol/vol) CAN–2.5% (vol/vol) trifluoroacetic acid–47.5% MilliQ water. As a positive control, 1 μl of Bacterial Test Standard solution (Bruker Daltonik, Bremen, Germany) was spotted twice and overlaid with HCCA matrix solution. Automatic runs were performed using flexControl version 3.3.108.0. Tested strains were identified by the MALDI Biotyper and MALDI Biotyper RTC software 3.0.

The current Bruker Daltonik database contains 4,110 main spectra (MSP). Ethanol extracts from 600 different yeast species of CBS-KNAW, including the set of strains of the *C. haemulonii* complex, were prepared and sent to Bruker Daltonik (Bremen, Germany) for MSP library creation according to the Bruker internal database creation standard operating procedures. This library was later uploaded at MALDI Biotyper 3.0 as an in-house CBS-KNAW library. This library could be used in combination with the Bruker database or as a stand-alone library.

For the MALDI-TOF MS-based recognition and classification of tested strains, MALDI Biotyper software 3.0 was used. In accordance with the manufacturer's specifications, the log (score) values demonstrating secure genus and species identification (≥2.0), secure genus identification (1.70 to 1.99), and no reliable identification (<1.7) were used. Bruker flex analysis 3.3.75.0 allowed visualization of the mass spectra acquired. ClinProTools 3.0 (Bruker Daltonik, Bremen, Germany) was used to generate artificial gel views of spectral peak intensities to search for differentiating peaks of these spectra and principal-component analysis clustering. Dendrograms were generated by using the respective functionality of the MALDI Biotyper 3.0 offline client. For the creation of dendrograms, the settings used were correlation as distance measure, ward linkage algorithm, and maximal clustering as 4 (four) and 0 (zero) as the maximal number of top-level nodes, and no cutoff (Co) values were used.

AFLP experiments. AFLP reactions were performed as described by Borst et al., with some minor modifications (3). In the second PCR, the selective primer MseI-G was used. Selective products were run at the Uppsala Genome Center (Uppsala, Sweden) using GeneScan-500 (6-carboxy-X-rhodamine labeled) as an internal size standard. The data were analyzed with the BioNumerics software package, version 4.61 (Applied Maths, Sint-Martens-Latem, Belgium) using the curve-based cosine similarity coefficient in combination with single-linkage cluster analysis to create the dendrogram.

Physiological tests. Morphological, biochemical, and physiological characteristics were examined as described by Kurtzman et al. (20). Fermentation, carbon assimilation, and nitrogen assimilation tests were done for 11 isolates that represented the three clades (i.e., CBS 5149, CBS 5150, CBS 7800, CBS 7799, CBS 7798, CNM-CL4640, CBS 6332, CNM-CL7239, CNM-CL7462, CNM-CL7256, and CNM-CL7073). Growth at different temperatures (25, 30, 35, 37, 40, and 42°C), in cycloheximide (0.01, 0.1%), on glucose (50 and 60%), and in NaCl (10, 16%) was also tested. Starch production, urease activity, the diazonium blue B staining reaction, and growth without vitamins were also investigated.

Morphology and mating. Thirteen isolates (CBS 5149, CBS 5150, CBS 7800, CBS 7799, CBS 7798, CNM-CL4640, CBS 6332, CNM-CL7239, CNM-CL7462, CNM-CL7256, CNM-CL7073, CBS 10913, and CBS 10004) were inoculated onto 5% yeast malt agar (YMA), GYPA, and morphology agar (MoA; Difco) and incubated for 12 days at 25°C to study colony morphology. To investigate cell morphology, strains were inoculated onto MoA plates and into yeast nitrogen base (YNB) medium con-

taining 5% glucose. Mating experiments were carried out with McClary acetate agar. Isolates in the same phylogenetic cluster were mixed two by two as follows: *C. haemulonii* group II, CBS 7799-CBS 7798, CBS 7799-CBS 7799, CBS 7799-CBS 7800, CBS 7800-CBS 7800, CBS 7800-CBS 7798, and CBS 7798-CBS 7798; four Spanish isolates of *C. haemulonii* with different ITS sequences, CNM-CL7256–CNM-CL7256, CNM-CL7256–CNM-CL7239, CNM-CL7256–CNM-CL6332, CNM-CL7256–CNM-CL7073, CNM-CL7256–CNM-CL7462, CNM-CL7239–CNM-CL7239, CNM-CL7239–CNM-CL6332, CNM-CL7239–CNM-CL7073, CNM-CL7239–CNM-CL7462, CNM-CL6332–CNM-CL6332, CNM-CL6332–CNM-CL7462, CNM-CL7073–CNM-CL7073, CNM-CL7073–CNM-CL7462, and CNM-CL7462–CNM-CL7462. Plates were incubated at 25°C for 40 days and viewed weekly by microscopy to check for the formation of ascospores.

Susceptibility tests. Susceptibility tests were done as recommended by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (EUCAST) for fermentative yeasts (9). All of the antifungal tests were evaluated after 48 h. Differences between 24-h and 48-h MIC values were no more than 1 2-fold dilution (data not shown). Slow growth of *C. haemulonii* strains has been described before (16). The antifungal agents used were AMB, flucytosine (5-FC; Sigma-Aldrich), FLC (Pfizer S.A., Madrid, Spain), itraconazole (ITC; Sigma-Aldrich), voriconazole (VRC; Pfizer S.A.), caspofungin (CAS; Merck & Co., Inc.), micafungin (MCF; Astellas Pharma, Inc., Tokyo, Japan), anidulafungin (ANF; Pfizer S.A), and posaconazole (PSZ; Merck & Co., Inc., Rahway, NJ). Interpretative breakpoints proposed by EUCAST for FLC were used (32, 33). For AMB, VRC, ITC, and PSZ, the breakpoints were defined on the basis of the wild-type distribution of MICs determined by the EUCAST method (epidemiological cut off) and on pharmacokinetic/pharmacodynamic and bibliographic data (8, 10, 31). In the case of echinocandins, breakpoints proposed by the EUCAST were used to interpret the susceptibility results (http://www.eucast.org/clinical_breakpoints/). Statistical and descriptive analyses of the MICs were also done, including the geometric mean (GM), range, and MIC_{90}. The significance of the differences between MIC values was determined by analysis of variance (Bonferroni *post hoc* test) (PASW statistics 18; IBM Software, Madrid, Spain). A P value of <0.01 was regarded as statistically significant.

Biofilm formation. Isolates of the *C. haemulonii* complex (Table 1) were used for biofilm formation. Additionally, strains of other *Candida* species that are known to form biofilms were selected for comparison, namely, *C. albicans* CBS 8758 (= SC 5314), *C. glabrata* CBS 861, *C. dubliniensis* CBS 7987, *C. krusei* CBS 573, and *C. tropicalis* CBS 8072. The standard protocol of Li et al. (25), with minor modifications, as reported by Kolecka et al. (19), was used. The ability to form biofilm was quantified by the crystal violet staining method according to Jin et al. (15). Final results were expressed as an average of three independent experiments ± the standard deviation (SD) where biofilms of each strain tested were cultivated in four parallel wells. The presence of biofilms was evaluated according to the Co value calculated from the average optical density at 600 nm (OD_{600}) of the three measurements of the negative control plus 3 times the SD as suggested by Holá et al. (14). Biofilm formation by each isolate was scored as negative (OD_{600} = <0.111 [Co value]), weak (OD_{600} = Co − [2 × Co]), intermediate (OD_{600} = [2 × Co] − [3 × Co]), or strong (OD_{600} = >3 × Co).

Nucleotide sequence accession numbers. The sequences obtained during this study were deposited in GenBank under the accession numbers listed in Table 2.

TABLE 2 Isolates used in this study and GenBank accession numbers of the genes sequenced

Isolate	GenBank accession no.			
	ITS	D1/D2	*RPB1*	*RPB2*
CNM-CL7256	JX459687	JX459788	JX459720	JX459754
CNM-CL7462	JX459688	JX459789	JX459721	JX459755
CNM-CL4642	JX459683	JX459784	JX459716	JX459750
CNM-CL3458	JX459680	JX459781	JX459713	JX459747
CNM-CL7829W	JX459691	JX459792	JX459724	JX459758
CNM-CL7239	JX459686	JX459787	JX459719	JX459753
CNM-CL7829P	JX459690	JX459791	JX459723	JX459757
CNM-CL6800	JX459684	JX459785	JX459717	JX459751
CNM-CL7793	JX459690	JX459790	JX459722	JX459756
CNM-CL4640	JX459681	JX459782	JX459714	JX459748
CNM-CL7073	JX459685	JX459786	JX459718	JX459752
CNM-CL4641	JX459682	JX459783	JX459715	JX459749
CBS 7800	JX459668	JX459767	JX459700	JX459733
CBS 7799	JX459667	JX459766	JX459699	JX459732
CBS 5149	JX459660	JX459759	JX459692	JX459725
CBS 9754	JX459671	JX459770	JX459703	JX459736
CBS 7801	JX459669	JX459768	JX459701	JX459734
CBS 5150	JX459661	JX459760	JX459693	JX459726
CBS 6590	JX459664	JX459763	JX459696	JX459729
CBS 5468	JX459662	JX459761	JX459694	JX459727
CBS 6915	JX459665	JX459764	JX459697	JX459730
CBS 7798	JX459666	JX459765	JX459698	JX459731
CBS 7802	JX459670	JX459769	JX459702	JX459735
CBS 6332	JX459663	JX459762	JX459695	JX459728
CBS 10973	JX459677	JX459777	JX459710	JX459743
CBS 10972	JX459676	JX459776	JX459709	JX459742
CBS 10971	JX459675	JX459775	JX459708	JX459741
CBS 10970	JX459674	JX459774	JX459707	JX459740
CBS 10969	JX459673	JX459773	JX459706	JX459739
CBS 10968	JX459672	JX459772	JX459705	JX459738
CBS 10004	AB118792	JX459771	JX459704	JX459737
KCTC-17807	JX459678	JX459778	NS[a]	JX459744
KCTC-17809	EU884177	JX459780	JX459712	JX459746
KCTC-17810	JX459679	JX459779	JX459711	JX459745

[a] NS, not sequenced.

RESULTS

Phylogeny. Two main clusters of the strains of the *C. haemulonii* complex were defined by sequence analysis of the four genes studied (ITS, D1/D2, *RPB1*, and *RPB2*). Twenty-three isolates were assigned to the most commonly encountered group, *C. haemulonii* group I, and seven isolates were assigned to *C. haemulonii* group II. In the ITS phylogenic tree (Fig. 1), four main clusters could be distinguished. The first cluster contained 19 *C. haemulonii* group I isolates and 4 isolates (CNM-CL7256, CNM-CL7462, CNM-CL7239, CNM-CL7073) that differ from the other 19 isolates showing 96% similarity to typical isolates of *C. haemulonii* group I. The percentages of similarity between the four isolates and the *C. haemulonii* type strain, CBS 5149^T, were as follows: 96.51% for CNM-CL7239 and CNM-CL7256, 96.31% for CNM-CL7462, and 96.43% for CNM-CL7073. The second cluster is formed by the seven isolates belonging to *C. haemulonii* group II. The third and fourth clusters contained *C. pseudohaemulonii* and *C. auris* isolates, respectively. The last two species were included for comparison.

D1/D2, *RPB1*, and *RPB2* phylogenic studies also revealed four main clusters. The first cluster included 23 *C. haemulonii* group I isolates, and the other cluster is formed by the 7 isolates belonging to *C. haemulonii* group II (see Fig. S1 to S3 in the supplemental material). *C. pseudohaemulonii* and *C. auris* isolates formed the third and fourth clusters, respectively. Strain KCTC 17807 was not included in the *RPB1* phylogenic tree because no good sequence of the *RPB1* gene was obtained, despite several tries. The sequence of

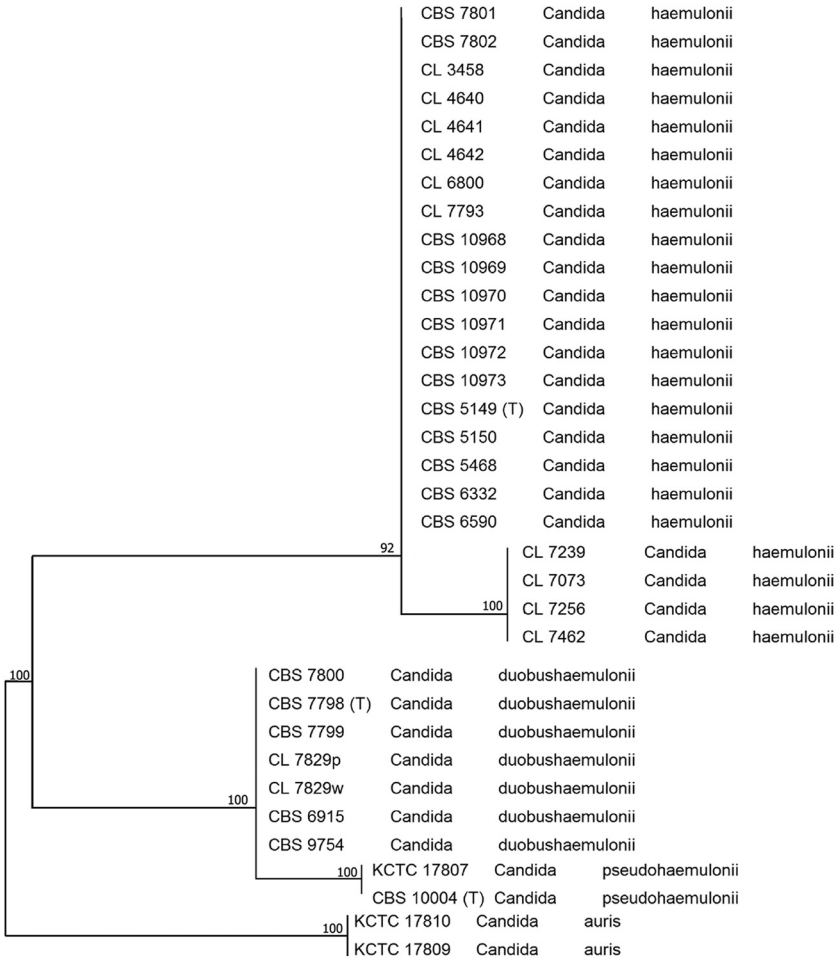

FIG 1 Phylogenetic tree of isolates of the *C. haemulonii* complex obtained by using maximum-likelihood phylogenetic analyses and 2,000 bootstrap simulations based on ITS sequences.

the *RPB2* gene obtained for strain CNM-CL7073 was too short to be aligned with the other sequences, and hence, this strain was not included in the *RPB2* phylogenic tree. Phylogenetic trees of these genes (see Fig. S1 to S3 in the supplemental material) indicated good bootstrap support (>90%) for all clusters.

The similarities between the type strain of *C. haemulonii* group I (CBS 5149^T) and the reference strain of *C. haemulonii* group II (CBS 7798) were 89.04% for ITS, 90.93% for D1/D2, 86.77% for *RPB1*, and 87.43% for *RPB2*.

Physiological tests, mating experiments, and biofilms. Some differences occurred in the fermentation tests, the carbon assimilation tests, growth at different temperatures, and growth in 60% glucose among *C. haemulonii* group II, *C. haemulonii* group I, and the *C. haemulonii* strains that differed in their ITS sequences (see Table S1 in the supplemental material). *C. haemulonii* group II is able to ferment raffinose, and it has differential patterns of carbon compound assimilation, growth temperatures, and growth in 60% glucose (Table 3). Differences between the two clades of the *C. haemulonii* complex (*C. haemulonii* type I and type II) are supported by their patterns of utilization of eight carbon compounds, namely, L-sorbose, arbutin, L-arabinose, L-arabinose, L-rhamnose, melezitose, inulin, and ethanol. The raffinose fermentation profiles differed as well (positive for *C. haemulonii* type II), as did growth at 37°C (negative for *C. haemulonii* type I) and growth in 60% glucose (positive for *C. haemulonii* type II). None of the mating experiments carried out gave any indication of the presence of a sexual cycle.

Biofilms that developed were quantified after 48 h (see Table S2 in the supplemental material). In general, strains of the *C. haemulonii* species complex do not form well-developed biofilms *in vitro* in YNB medium supplemented with 50 mM glucose.

Susceptibility tests. The 30 isolates were tested for susceptibility to nine antifungal compounds (see Materials and Methods). The MICs of AMB ranged from 0.25 to >16 mg/liter for all 30 isolates of the *C. haemulonii* complex. *C. haemulonii* group II and the four strains of *C. haemulonii* that showed deviant ITS sequences had high MICs of AMB (GM, 2.0 mg/liter and 1.18 mg/liter, respectively) and azoles (Table 4). Statistically significantly higher AMB MICs were found for *C. duobushaemulonii* ($P = 0.008$).

All of the isolates of the new variety, 6 out of 7 isolates of *C. haemulonii* group II, and 6 out of 19 isolates of *C. haemulonii* group I showed high MICs of AMB (Table 4). The MIC ranges of FLC, ITC, VRC, and PSZ for all 30 isolates were 8 to >64 mg/liter, 0.06 to >8 mg/liter, 0.12 to >8 mg/liter, <0.015 to >8 mg/liter, and 0.015 to >8 mg/liter, respectively. It is noteworthy that 28 out

TABLE 3 Differential characteristics of the taxa of the *C. haemulonii* complex[a]

Characteristic	*C. haemulonii* CBS 5149, CBS 5150, CNM-CL4640, CBS 6332	*C. duobushaemulonii* CBS 7798, CBS 7799, CBS 7800	*C. haemulonii* var. *vulnera* CNM-CL7239, CNM-CL7462, CNM-CL7256, CNM-CL7073	*C. auris*[b]	*C. pseudohaemulonii*[b]
Fermentation of:					
Raffinose	−	+	−	−	−
Sucrose	+	+	+	+	−
Assimilation of:					
L−Sorbose	−	+	−	−	+
Arbutin	−	+	−	ND	ND
L-Arabinose	−	W	−	−	V
D-Arabinose	−	V	−	−	−
L-Rhamnose	+	W	+	−	+
Melezitose	+	+	D	+	+
Inulin	−	+	W	W	−
Ethanol	D	W/D	+	−	D
D-Galactose	+	+	+	−	+
Methanol	W/D	W/D	W/D	−	−
Succinate	+	+	+	−	+
D-Gluconate	+	+	+	−	+
Xylitol	W/D	+	+	−	+
Glycerol	+	+	+	−	+
Growth at:					
37°C	−	+	+	+	+
40°C	−	−	−	+	−
Growth in:					
60% glucose	−	+	−	−	ND
Vitamin-free medium	−	−	−	+	ND

[a] +, positive; −, negative; W, weak reaction; D, delayed reaction; W/D, weak and delayed; ND, not determined; V, variable.
[b] *C. auris* and *C. pseudohaemulonii* data are from references 35 and 38, respectively.

of 30 isolates could be considered cross-resistant to azoles. Only 2 out of 30 isolates with FLC MICs of >4 mg/liter showed low MICs of the other azole compounds (Table 4). In general terms, there were subtle observed differences in azole MICs between the groups of strains tested, showing higher MICs for *C. haemulonii* than for the other two groups. In some cases (MICs of ITC and VRC for *C. haemulonii* and *C. duobushaemulonii*), the differences were significant or close to significance ($P = 0.009$ and $P = 0.028$, respectively).

Strains with high MICs of CAS, ANF, and MCF were found as well. Three out of four isolates of the ITS variant of *C. haemulonii* showed high MICs of CAS, and one of them (CNM-CL7239) showed high MICs of all of the echinocandins studied. Fifteen out of 19 isolates of *C. haemulonii* showed high MICs of CAS, and 4 out of these 15 isolates showed the same profile for all echinocandins. With respect to *C. haemulonii* group II, four out of seven isolates showed high MICs of CAS. However, this susceptibility profile was totally different for ANF or MCF (Table 4).

Identification of species. Representatives of *C. haemulonii* and *C. duobushaemulonii* can be identified by ITS sequencing, which gives reliable identification of *C. haemulonii*, the ITS variant of *C. haemulonii*, *C. duobushaemulonii* sp. nov., *C. auris*, and *C. pseudohaemulonii* (Fig. 1).

MALDI-TOF MS also identified all of the species, but here the ITS variant of *C. haemulonii* clustered among typical *C. haemulonii* strains (Fig. 2). MALDI-TOF MS correctly identified 32 (86.5%) of 37 isolates with log scores of ≥2.0 for at least one spot and 5 (13.5%) of 37 with log scores of 1.700 to 1.999 on one spot (see Table S3 in the supplemental material). The artificial gel view acquired from the type strains illustrated differences in mass spectral peak positions and intensities which are species unique (see Fig. S4 in the supplemental material). The technical variation between duplicates of the same strains obtained from different culture collections are illustrated by *C. pseudohaemulonii* CBS 1004^T and JCM 12453^T and *C. auris* JCM 15448^T and DSM 21092^T.

The AFLP experiments showed the same strain grouping as the MALDI-TOF MS analyses (Fig. 3), and here the strains of *C. haemulonii* with a deviating ITS sequence could not be distinguished from typical strains of *C. haemulonii*.

Taxonomy. *Candida duobushaemulonii* sp. nov. E. Cendejas-Bueno, A. Kolecka, A. Alastruey-Izquierdo, A. Gómez-López, M. Cuenca-Estrella, and T. Boekhout.

MycoBank: "*Candida duobushaemulonii*" (MB 800921).

Etymology: the epithet *duobushaemulonii* "*haemulonii* two" refers to the previous name by which this species was known, *C. haemulonii* group II.

In 5% glucose liquid broth, at 25°C for 72 h, cells are subspherical to ellipsoidal, 2.5 to 5 μm by 2 to 3.5 μm (Fig. 4), and occur singly, in pairs, or in small clusters, with multipolar or unipolar budding. No filaments or hyphae are formed on MoA. On GYPA

TABLE 4 Antifungal susceptibility profiles of the 30 isolates in this study

Strain or parameter	MIC (mg/liter)								
	AMB	5-FC	FLC	ITC	VRC	PSZ	CAS	MCF	ANF
C. haemulonii var. *vulnera*									
CNM-CL7239	1	<0.12	>64	>8	>8	>8	>16[a]	>16[a]	>16[a]
CNM-CL7256	2	0.5	>64	>8	>8	>8	>16[a]	0.12	0.06
CNM-CL7073	1	0.25	>64	>8	>8	>8	0.5[a]	0.12	0.06
CNM-CL7462	1	0.5	>64	>8	>8	>8	>16[a]	0.06	<0.03
GM (range)	1.18 (1.0–2.0)	0.24 (0.06–0.5)	128 (>64)	16 (>8)	16 (>8)	16 (>8)	11.31 (0.5–16)	0.40 (0.06–>16)	0.20 (<0.03–>16)
MIC_{90}	1.7	0.5	128	16	16	16	32	22.436	22.418
C. duobushaemuloni									
CBS 7798	4	0.5	>64	4	>8	>8	>16[a]	0.06	4
CBS 6915	2	0.12	8	0.12	0.25	0.015	>16[a]	0.06	0.03
CBS 7800	0.25	0.25	>64	>8	>8	>8	>16	0.06	0.25
CBS 7799	1	2	>64	>8	>8	>8	16	0.06	0.06
CBS 9754	1	0.25	>64	>8	>8	>8	1	0.12	0.06
CNM-CL7829W	>16	<0.125	>64	>8	>8	>8	0.5	0.06	<0.03
CNM-CL7829P	2	0.25	16	0.06	0.12	0.03	0.5	0.06	<0.03
GM (range)	2 (0.25–32)	0.27 (0.06–2)	64 (8–>64)	2.93 (0.06–>8)	4.39 (0.12–>8)	2.18 (0.015–>8)	5.38 (0.5–>16)	0.06 (0.06–0.12)	0.08 (<0.03–4)
MIC_{90}	15.2	1.1	128	16	16	16	32	0.084	1.75
C. haemulonii									
CBS 7802	1	0.25	>64	>8	>8	>8	>16[a]	>16[a]	0.25
CBS 7801	1	0.25	>64	>8	>8	>8	0.25[a]	0.12[a]	<0.03
CBS 6332	0.25	0.5	>64	>8	>8	>8	>16	>16[a]	4
CBS 10970	1	1	>64	>8	>8	>8	0.25[a]	0.12[a]	<0.03
CBS 6590	0.5	<0.12	>64	>8	>8	>8	>16[a]	0.06	<0.03
CBS 5150	0.5	<0.12	>64	>8	>8	>8	>16[a]	0.06	<0.03
CBS 5468	0.5	0.25	>64	>8	>8	>8	>16[a]	>16[a]	>16[a]
CBS 5149	0.25	<0.12	>64	>8	>8	4	0.25	<0.03	<0.03
CBS 10972	0.5	0.5	>64	>8	>8	>8	>16[a]	0.06	<0.03
CBS 10971	0.5	1	>64	>8	>8	8	>16[a]	0.06	<0.03
CBS 10969	0.5	1	>64	>8	>8	8	>16[a]	0.06	<0.03
CBS 10968	0.5	2	>64	>8	>8	>8	>16[a]	0.06	<0.03
CBS 10973	0.5	0.25	>64	>8	>8	>8	>16[a]	0.06	0.06
CNM-CL7793	2	0.25	>64	>8	>8	>8	>16[a]	0.06	<0.03
CNM-CL3458	0.5	<0.12	>64	>8	>8	>8	>16[a]	0.25	0.12
CNM-CL4640	0.25	<0.12	>64	>8	>8	0.03	8[a]	<0.03	<0.03
CNM-CL4641	1	<0.12	>64	>8	>8	>8	>16	>16	>16
CNM-CL4642	0.5	0.25	>64	>8	>8	>8	0.5	0.06	<0.03
CNM-CL6800	1	0.12	>64	>8	>8	>8	>16[a]	0.06	<0.03
GM (range)	0.57 (0.25–2)	0.22 (0.06–2)	128 (>64)	16 (>8)	16 (>8)	10.68 (0.03–>8)	11.10 (0.25–>16)	0.17 (<0.03–>16)	0.06 (<0.03–>16)
MIC_{90}	1	1	128	16	16	16	32	32	9.6

[a] Strain that showed some kind of paradoxical growth effect.

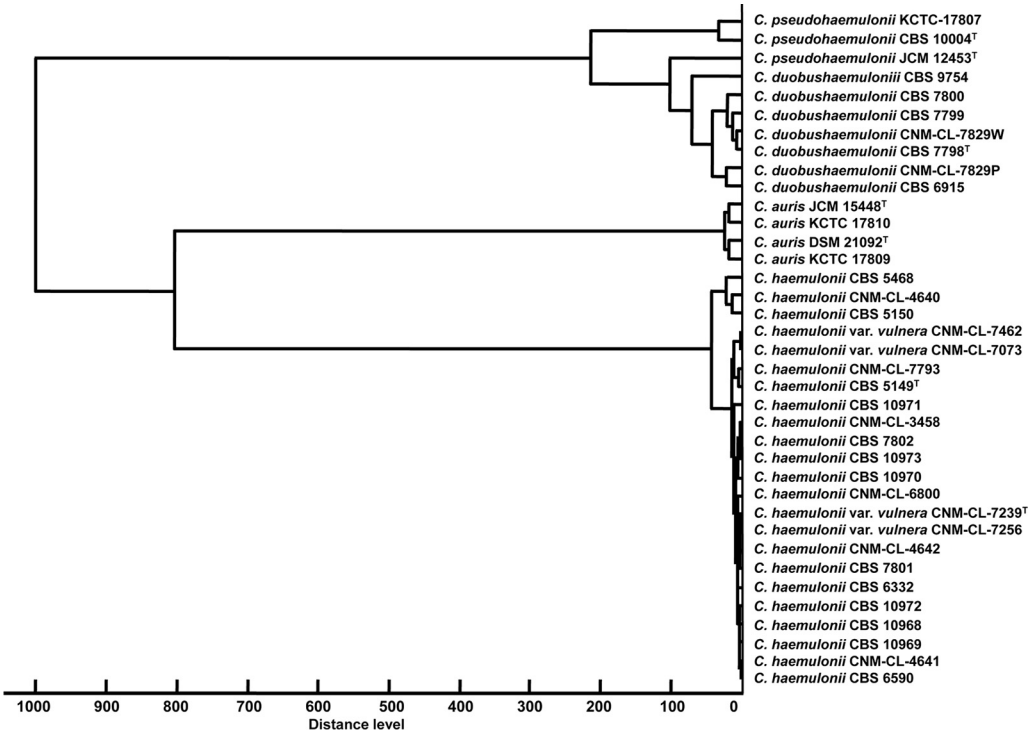

FIG 2 Dendrogram clustering the MALDI-TOF MSP obtained from at least 20 mass spectra of strains belonging to the *C. haemulonii* complex species and related species. *C. auris* JCM 15448T and DSM 21092T and *C. pseudohaemulonii* JCM 12453^T were added to make the sampling in MALDI-TOF MS more robust.

and YMA after 12 days of growth, the colonies are about 1 cm in diameter, creamy white, and smooth, with a central ring and weak grooves that run from the center of the colony to the entire margin. For descriptions of its biochemical and physiological features, see Table 3; see also Table S1 in the supplemental material. The holotype, CBS 7798 (CNM-CL9157), was isolated from a human foot ulcer in Alabama. Other representative isolates are CBS 7800 (CNM-CL9179; foot ulcer, United States), CBS 7799 (CNM-CL9178; foot ulcer, United States), CBS 9754 (CNM-CL9165; insect, Germany), CBS 6915 (CNM-CL9167; source unknown, country unknown), CNM-CL7829P, and CNM-CL7829W (blood, Spain). These strains have also been deposited in the Centro Nacional de Microbiología (CNM) collection.

Candida haemulonii var. *vulnera* var. nov. E. Cendejas-Bueno, A. Kolecka, A. Alastruey-Izquierdo, A. Gómez-López, M. Cuenca-Estrella, and T. Boekhout.

MycoBank: "*Candida haemulonii vulnera*" (MB 800922).

Etymology: the epithet *vulnera* "wound" refers to a clinical sample from which the type strain was isolated.

Differs from the typical variety (*C. haemulonii*) on the basis of phenotypic, susceptibility, and molecular features, namely, delayed assimilation of melezitose, weak growth on inulin, growth on ethanol and xylitol, and growth at 37°C. The AMB MIC is higher than that for *C. haemulonii*, and the ITS sequence differs from the typical *C. haemulonii* ITS sequence, showing 96% similarity to typical isolates of *C. haemulonii* group I.

The holotype, CNM-CL7239, was isolated from a human skin wound in León, Spain. This strain has been deposited in the Col-

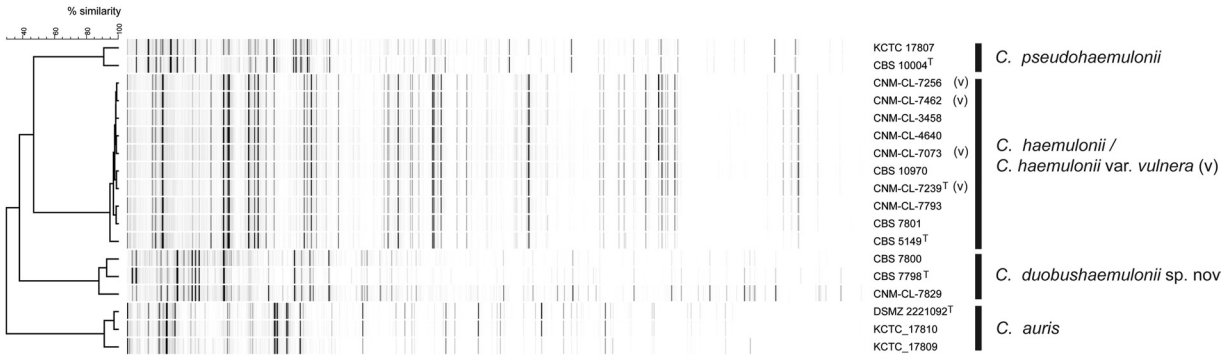

FIG 3 AFLP patterns of representative isolates of the *C. haemulonii* complex and related species.

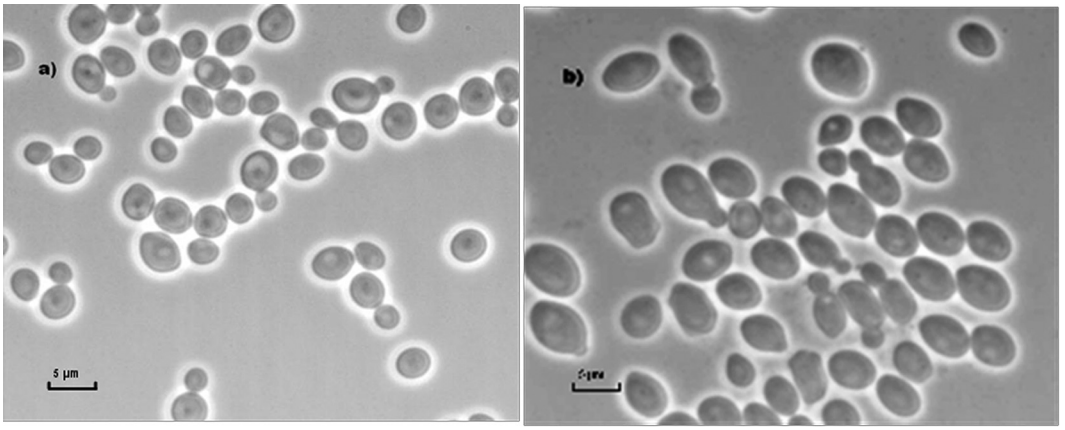

FIG 4 Cell morphology of *C. duobushaemulonii* sp. nov. on MoA (a) and in 5% glucose liquid broth (b).

lection of the CNM Mycology Department. Other isolates are CNM-CL-7256 (toenail, Alicante, Spain), CNM-CL-7462 (source unknown from a human clinical sample, Spain), and CNM-CL-7073 (skin wound, Alicante, Spain). They have also been deposited in the CBS collection under the numbers CBS 12439 (CNM-CL7239), CBS 12436 (CNM-CL7256), CBS 12437 (CNM-CL 7073), and CBS 12438 (CNM-CL 7462).

DISCUSSION

Because of advances in molecular taxonomy, many species of yeasts that can cause infections in humans have been described recently. Some of these new pathogens (e.g., *C. orthopsilosis*, *C. metapsilosis*, *C. nivariensis*, and *C. bracarensis*) have been well characterized by molecular methods, such as PCR-based procedures and sequence analysis (amplification of ITS regions and the D1/D2 domain and analysis of sequence polymorphisms) (1, 35, 38, 39). Several reports have addressed the difficulty of identifying rare yeast isolates to the species level by conventional methods, since they are highly dependent on variables such as the growth medium and temperature (4). In addition, databases of commercial identification systems are limited to the species commonly found in the clinic, and in general terms, their use is time-consuming. On the other hand, molecular methods based on DNA sequencing resulted in the improved characterization of strains (4). Because of the emergence of resistant yeast pathogens, it is important that the available identification methods provide the highest possible degree of precision. Furthermore, providing reliable antifungal susceptibility profiles of these rare pathogenic yeasts will improve the management of patients with fungal infections due to these organisms.

In 1993, Lehman et al. described two different groups among *C. haemulonii* isolates (24) and concluded that these isolates represented a species complex. This classification is still in place today. In this study, we demonstrated that the *C. haemulonii* complex comprises two species, *C. haemulonii* group I and *C. haemulonii* group II, using phenotypic and molecular methods. They differ from each other in the four genes sequenced, some phenotypic features, and MALDI-TOF MS and AFLP profiles. Surprisingly, we found four *C. haemulonii* group I isolates that differ in the ITS gene sequences (96% similarity to the *C. haemulonii* group I type strain ITS sequences), whereas the other three genes studied were identical. The four strains also differ in some physiological features from *C. haemulonii* group I, including assimilation of melezitose and inulin and growth at 37°C. However, they did not differ by proteomics and AFLP analysis, and hence, we concluded that these four isolates represent a variety of *C. haemulonii*.

Only one study included a strain of *C. haemulonii* CBS 5149^T (type strain) for MALDI-TOF MS testing (29). Here we present data on MALDI-TOF MS validation for fast and accurate identification of strains and clinical isolates of the species studied. MALDI-TOF MS allowed differentiation among all of the species in the *C. haemulonii* complex, producing secure species identification because all of the isolates could be identified to the species level with a reliability threshold of log scores of >1.7. The MALDI-TOF MS results were congruent with those obtained by AFLP typing.

Susceptibility tests of all of the strains included in this work established differences in their antifungal susceptibility patterns. In previous studies, all isolates of *C. haemulonii* were found to be resistant to both AMB and FLC (MIC range, 6.16 to 18.30 mg/liter). However, we found that our isolates from human, animal, and environmental sources demonstrated variable patterns of susceptibility to AMB (MIC range, 0.25 to >16 mg/liter) and FLC (MIC range, 8 to >64 mg/liter). Most of the FLC-resistant strains appeared to demonstrate azole cross-resistance (MIC of ITC, 4 to >8 mg/liter; MIC of VRC, >8 mg/liter; MIC of PSZ, >8 mg/liter), which was not described in previous reports, where most of the strains were reported to be susceptible to VRC and PSZ (16, 17, 34). Both *C. haemulonii* groups I and II have been reported to be susceptible to ANF and MCF (6, 17, 18, 34). In this study, the MICs of both MCF and ANF for all 30 isolates ranged from <0.03 to >16 mg/liter, thus showing that these two echinocandins are active against most of the 30 isolates in our collection. Some isolates, however, show high MICs of CAS, and some had a profile of cross-resistance to the three echinocandins, which has not been described before (MIC range, 16 to 18.34 mg/liter). When *Candida* spp. are grown in a medium containing a high concentration of an antifungal agent such as CAS, the result can be reduced activity of that agent against certain organisms. This phenomenon is called the Eagle effect or the paradoxical growth effect. The Eagle effect has previously been reported in many *Candida* species (12). In accordance with the results obtained with the *C. haemulonii* complex and the three echinocandins tested, more studies should

be done to clarify the role of this paradoxical effect on the profile of susceptibility of these yeasts to these compounds.

With respect to biofilm formation, our data could not demonstrate significant differences between various species of the *C. haemulonii* complex. Recently, Oh et al. (28) reported that strains of *C. haemulonii* and *C. pseudohaemulonii* isolated from blood cultures showed good biofilm formation compared to *C. auris* isolates from ear specimens that did not form biofilms. The authors also correlated the extensive biofilm production with the origin of the isolates. Strains from central venous catheter-related fungemia recovered from patients receiving total parenteral nutrition did prominently form biofilms, whereas strains from ear specimens did not (28). In our study, the YNB medium was supplemented with 50 mM glucose (0.9%), which is the recommended medium for standard evaluation of biofilm formation (25, 28). In contrast, Shin et al. (37) used Sabouraud's dextrose broth supplemented with 8% glucose. These authors described differences in the ability to form biofilm among different *Candida* species cultivated in this high-glucose (8%) medium.

Here we propose a reclassification of the *C. haemulonii* complex and describe former *C. haemulonii* group II as a new species. We consider the differences in the DNA sequences of several genes among strains of *C. haemulonii* groups I and II to be too great to consider them conspecific, with similarity values found between the type strain of *C. haemulonii* group I (CBS 5149^T) and the reference strain of *C. haemulonii* group II (CBS 7798) of 89.04% for ITS, 90.93% for D1/D2, 86.77% for *RPB1*, and 87.43% for *RPB2*. The different patterns of the two species in the MALDI-TOF MS and AFPL experiments corroborate the results of the molecular study. Differences in phenotypic and susceptibility features have also been observed. Different profiles of fermentation of raffinose and sucrose and assimilation of carbon compounds (for instance, L-sorbose, inulin, D-galactose, and succinate), different growth temperatures, and other discordant features were found. These different features could help to distinguish between the two species of the *C. haemulonii* species complex in the clinical setting when a molecular method or MALDI-TOF MS is not available. For this species, we propose the name *C. duobushaemulonii* sp. nov. (synonym: *C. haemulonii* group II).

In addition, a variety of *C. haemulonii* is described as *C. haemulonii* var. *vulnera*, which differs from other *C. haemulonii* isolates in the ITS sequence, some physiological and biochemical features, and its AMB susceptibility profile. Because we have only four isolates that probably accidentally were all obtained in Spain, we do not feel comfortable in commenting on this observation yet. If it turns out in the future that this variety is dominant in southern Europe or somewhere else, it may warrant some further mention.

The new taxa described here have a multiantifungal resistance profile that includes high MICs of AMB and cross-resistance to azole compounds and impairs the treatment of infections with these species with echinocandins and 5-FC. This fact is important in order to establish the correct treatment of patients with fungal infections due to these yeasts.

Fungemia due to *C. haemulonii* and its closely related new species is rare. Reliable identification is needed to start appropriate treatment and provide optimal management of infections due to these yeasts. Classical methods of identification are not able to identify these rare clinical isolates. Importantly, most of the uncommon yeasts that are incorrectly identified using conventional methods showed an antifungal resistance profile (i.e., *C. haemulonii*, *Candida ciferri*, *Pichia anomala*, *Pichia membranifaciens*, *Pichia fermentans*, *Kodamaea ohmeri*, and *Candida rugosa*) (4), and such incorrect identifications lead to inappropriate treatment and clinical management. *C. haemulonii*, *C. haemulonii* var. *vulnera*, *C. duobushaemulonii*, *C. auris*, and *C. pseudohaemulonii* are part of this group of rare clinical yeast that so far cannot be well differentiated by current commercial methods (17, 23, 30, 34). Identification of these species should be based on molecular methods such as PCR, sequencing analyses, and MALDI-TOF MS analyses. According to the results of sequencing and MALDI-TOF MS, we conclude that the best clinical identification at the species and variety levels can be done with the molecular barcode proposed for fungal identification, namely, the ITS region (36) and MALDI-TOF MS.

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